

## THE DOUBLE STAINING OF SPORES AND BACILLI.

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TO THE SOCIETY.

Until quite recently the methods recommended by various authors for staining the spores of bacilli produced results that were far from being satisfactory. Fortunately this state of affairs has been removed by Klein\*, of Amsterdam, who has published a method which, with a little improvement, is eminently successful.

The methods in general use consist in the first place in preparing a film of the material to be stained by mixing a portion of an agar or potato culture of the bacillus with a drop of water upon a clean cover-glass and allowing the suspension to dry. The dried film is then fixed by passing it three times through the bunsen-flame, a process which has for its object the coagulation of the bacterial protoplasm and the attachment of the bacteria to the glass. From this point the methods differ. The spore-case or capsule which prevents the entrance of the stain within the spore is destroyed more or less by attacking it with physical or chemical agents. The physical treatment consists in heating the film in the air-bath for about half-an-hour at 200° C., or in the autoclave at 120° C., or even by passing the film many times through the bunsen-flame. The chemical manipulation involves the treatment of the film with strong sulphuric acid, potassium hydrate, zinc chloriodide, 5% chromic acid or 1½% hydrochloric acid. These methods are employed to soften the spore-capsule after it has been hardened by the process of fixation. Klein had

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\* Centralblatt für Bakteriologie, i. Abt. xxv. 376.

noted that it was much easier to kill bacteria and spores when they were in the moist condition than when they had been dried, and from this he reasoned that it would be much easier for the stain to get through the moist than through the dried spore-case. Accordingly he stained first and fixed afterwards.

His procedure is as follows:—A platinum loop is filled with a culture of the bacillus grown upon potato for 24 hours (at  $37^{\circ}$  C.), and this is introduced into a small quantity—say, two or three drops—of normal saline contained in a watch-glass. The culture is stirred in the saline until a homogeneous suspension is obtained. An equal number of drops of carbol-fuchsin is added, and the two fluids are thoroughly mixed. The watch-glass is placed high over a micro-chemical burner so that the heat applied is just sufficient to cause a slight vapour to hover over the surface of the fluid. A larger watch-glass is placed over the first to keep out the dust and to enable one to judge the intensity of the heating. There should be just a slight film of condensed water upon the covering glass. At the end of *six* minutes the watch-glass is taken from the burner and allowed to cool for a few moments. The bacilli and spores which have precipitated more or less are again distributed in the stain by imparting a rotatory motion to the cover-glass. A loop of the suspension is spread over a clean cover-glass and allowed to dry in the air. The film is fixed by passing it twice through the flame. The bacilli are then decolorised by immersing the cover-glass in 1% sulphuric acid for one or two seconds, after which the acid is washed off with water and the bacilli counter-stained in dilute watery-alcoholic methylene-blue for three or four minutes. The film is washed in water, dried and mounted in balsam. After this treatment the spores appear red, the bacilli blue.

The method as recommended by Klein is excellent as far as the principle is concerned, but the details might be altered with advantage. Some spores, instead of staining red, show only a pink margin. Klein does not push the staining process far enough, and indeed it is hardly possible to do so when a watch-

glass is used to hold the fluid. There are spores, as, for example, *Bac. lactis XII.* (Flügge), which stain quite readily with carbol-fuchsin in the usual manner after fixation by heat, while others, as a *Bac. leptosporus* sp., stain but faintly when Klein's method is employed. The refractory spores are stained a deep red by the following method:—Four drops of normal saline are pipetted into a small test-tube, and the spore-bearing material is rubbed up with this until a homogeneous suspension is obtained. Four drops of fresh carbol-fuchsin are pipetted into the tube and the mixture shaken. A plug of cotton wool is inserted and the tube placed into a beaker of boiling water. The water is boiled for a quarter of an hour, when the tube is taken out and shaken. A loopful of the bacterial suspension is withdrawn and spread uniformly over a cover-glass which is dried either in the air or high over a bunsen-flame. The film is next fixed by passing the cover-glass three times through the flame in the usual manner. The bacilli are decolorised in methylated spirit containing 1·5% (by volume) of concentrated hydrochloric acid. When the film appears colourless, the cover-glass is withdrawn and moved about in water to remove the alcohol, after which the film is stained with carbol-methylene-blue in the ordinary manner; it is then washed, dried and mounted.

The acidified alcohol appears to give a cleaner film than when sulphuric acid is employed. Klein apparently means 1% sulphuric acid by volume—that is, 1 c.c. of strong sulphuric acid to 99 c.c. of water; 1% by weight of sulphuric acid does not decolorise the film sufficiently. The spores of some of the water-bacilli are so readily decolorised by acidified alcohol that it is advisable to extract the excess of colour with methylated spirit, then to dip the cover-glass for a moment only into the acidified alcohol and quickly place in water.

Generally speaking, spores can be recognised by the fact that they are not coloured by the usual stains, and that the remains of the mother-cell stain sufficiently to enable one to make out the regular shape of the unstained spore. There occur cases, however,

when the investigator might easily be led astray. Certain rod-shaped water-bacteria become vacuolated as they grow old; the protoplasm aggregates at the poles, which stain deeply, leaving the centre of the rod unstained. In these cases the central unstained vacuole, and especially when it is oval, presents an appearance similar to an unstained spore. When counter-staining is made use of, the central vacuole does not stain. It is here that perhaps the advantage of possessing a process which stains the most refractory spores is evident. One can depend upon the spore being stained. In the case of vacuoles, it is possible, by limiting the decolorisation with acidified alcohol, and by counter-staining with blue, to obtain the bacteria with their dense terminal protoplasm stained red, and the vacuole pale blue.